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Human glutathione S-transferases- and NAD(P)H:quinone oxidoreductase 1-catalyzed inactivation of reactive quinoneimines of amodiaquine and N-desethylamodiaquine: Possible implications for susceptibility to amodiaquine-induced liver toxicity

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ABSTRACT

Amodiaquine (AQ), an antimalarial drug, widely prescribed in endemic areas of Africa and Asia, is used in combination with artesunate as recommended by the WHO. However, due to its idiosyncratic hepatotoxicity and agranulocytosis, the therapeutic use has been discontinued in most countries. Oxidative bioactivation to protein-reactive quinonimines (QIs) by hepatic cytochrome P450s and myeloperoxidase (MPO) have been suggested to be important mechanisms underlying AQ idiosyncratic toxicity. However, the inactivation of the reactive QIs by detoxifying enzymes such as human glutathione S-transferases (GSTs) and NAD(P)H:quinone oxidoreducatase 1 (NQO1) has not been characterized yet. In the present study, the activities of 15 recombinant human GSTs and NQO1 in the inactivation of reactive QIs of AQ and its pharmacological active metabolite, N-desethylamodiaquine (DEAQ) were investigated. The results showed that GSTP1-1, GSTA4-4, GSTM4-4, GSTM2-2 and GSTA2-2 (activity in decreasing order) were active isoforms in catalyzing GSH conjugation of reactive QIs of AQ and DEAQ. Additionally, NQO1 was shown to inactivate these QIs by reduction. Simulation of the variability of cytosolic GST-activity based on the hepatic GST contents from 22 liver donors, showed a large variation in cytosolic inactivation of QIs by GSH, especially at a reduced GSH-concentration. In conclusion, the present study demonstrates that a low hepatic expression of the active GSTs and NQO1 may increase the susceptibility of patients to AQ idiosyncratic hepatotoxicity.

1. Introduction

The antimalarial drug amodiaquine (AQ) has been widely used in the endemic areas of Africa and Asia over the last 50 years. WHO has catalogued the combination therapy of artesunate/amodiaquine as one of the strongest recommendation for the treatment of uncomplicated *P. falciparum* malaria (WHO, 2015). However, AQ is reported to cause severe idiosyncratic hepatotoxicity and agranulocytosis with an incidence of 0.05% among the patients (Larrey et al., 1986; Neftel et al., 1986; Hatton et al., 1986). In humans, AQ is rapidly metabolized to its principle metabolite N-desethylamodiaquine (DEAQ), predominantly by CYP2C8 (Li et al., 2002). Besides the formation of stable metabolites, AQ has been shown to be bioactivated to amodiaquine quinoneimine (AQ-QI) by several human cytochrome P450s (CYPs), including CYP3A4, CYP2D6, and CYP2C8 (Zhang et al., 2017). Additionally, the major metabolite DEAQ is also prone to bioactivation, to N-desethylamodiaquine quinoneimine (DEAQ-QI), by CYP2D6, CYP3A4, CYP2C8, and CYP2C9. Both protein-reactive quinoneimines (QIs) are considered

Abbreviations: AQ, amodiaquine; AQ-QI, amodiaquine quinoneimine; AQ-SG, 5′-glutathion-*S*-yl-amodiaquine; bis-DEAQ, N-bisdesethylamodiaquine; CDNB, 1-chloro-2,4-dinitrobenzene; CYP, cytochrome P450; DCPIP, 2,6-dichlorophenolindophenol; DEAQ, N-desethylamodiaquine; DEAQ-QI, N-desethylamodiaquine quinoneimine; DEAQ-SG, 5′-glutathion-*S*-yl-N-desethylamodiaquine; EDTA, 2,2′,2″,2‴-(ethane-1,2-diyldinitrilo)tetraacetic acid; EPNP, 1,2-epoxy-3-(*p*-nitrophenoxy)propane; GSH, glutathione; GST, glutathione S-transferase; HLM, human liver microsomes; IDILI, idiosyncratic drug-induced liver injury; IPTG, isopropyl β-D-1-thiogalactopyranoside; $MgCl_2$, magnesium chloride; MPO, myeloperoxidase; NADPH, β-nicotinamide adenine dinucleotide 2′-phosphate; NAPQI, N-acetyl-*p*-benzoquinonimine; NRS, NADPH regenerating system; NQO1, NAD(P)H quinone oxidoreducatase 1; ORF, open reading frame; QI, quinoneimine; SDS-PAGE, sodium dodecyl sulfate polyacrylamide gel electrophoresis

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the main reactive metabolites which may play a role in AQ-induced idiosyncratic toxicity (Harrison et al., 1992). Since the inter-individual variation of these CYP isozymes is very large among populations (Achour et al., 2014), large variability in internal exposure to AQ-QI and DEAQ-QI can be expected.

Besides the bioactivation, inactivation of reactive metabolites by glutathione (GSH) and inactivating enzymes, such as human glutathione *S*-transferases (GSTs) and NAD(P)H:quinone oxidoreductase 1 (NQO1) are also important factors determining the internal exposure of tissues to reactive metabolites. For example, Tafazoli and O'Brien (2009) found that depletion of cellular GSH caused significantly increased AQ cytotoxicity in rat hepatocytes. Shimizu et al. (2009) demonstrated that covalent protein binding and hepatotoxicity was significantly increased in GSH-depleted rats when compared to control rats after AQ treatment. Previous studies also showed that AQ-QI and DEAQ-QI are inactivated by GSH, forming the corresponding GSH conjugates 5′-glutathione-*S*-yl-amodiaquine (AQ-SG) (Harrsion et al., 1992) and 5′-glutathione-*S*-yl-N-desethylamodiaquine (DEAQ-SG) (Zhang et al., 2017), respectively. GSTs may play important roles in the detoxification of electrophilic substrates, especially in cases electrophiles have a low direct reactivity towards GSH. GSTs predominantly catalyze the conjugation reactions of reduced GSH to electrophilic compounds, but also the reduction of organic hydroperoxides, thereby preventing cytotoxicity or mutagenicity (Jancova et al., 2010). It has been shown that GST isoenzymes catalyze the inactivation of reactive metabolites of many drugs, such clozapine (Dragovic et al., 2010), diclofenac (Dragovic et al., 2013; Den Braver et al., 2016), mefenamic acid (Venkataraman et al., 2014), and nevirapine (Dekker et al., 2016).

Besides GSTs, also the human NQO1 is capable of detoxifying QIs, via two-electron reduction (Kucera et al., 2013). A recent study showed that NQO1 catalyzes the inactivation of reactive QIs of diclofenac, mefenamic acid, and acetaminophen (Vredenburg et al., 2014), and the efficacy of inactivation was even stronger compared to the most active GST isoforms towards certain reactive QIs. Moreover, Tafazoli and O'Brien (2009) demonstrated that both cytotoxicity and carbonylation in rat hepatocytes were significantly increased by inhibition of NQOs with dicoumarol upon AQ treatement, which also supports the protective role of NQOs against AQ cytotoxicity in a cellular context. However, direct evidence proving NQO1's role in detoxifying reactive QIs of AQ and DEAQ is still missing.

Because both human GSTs and NQO1 show large inter-individual variability in expression levels (Jancova et al., 2010; Jaiswal, 2000), individuals with low enzyme activity might be more susceptible towards the same internal exposure of reactive metabolites. In case of GSTs, the null genotypes of GSTM1 and GSTT1, resulting in no expression levels, have been reported as a risk factor in idiosyncratic drug-induced liver injury (IDILI) caused by NSAIDs with an odds-ratio of 8.8 (Lucena et al., 2008). For NQO1, polymorphic alleles NQO1*2 and NQO1*3 have been identified and individuals carrying the NQO1*2 allele showed very low expression levels compared to that of the NQO1*1 allele (Siegel et al., 2012; Moran et al., 1999). Moreover, a recent study showed that the expression levels of NQO1 in human livers varied with ethnicity and obesity factors (Rougée et al., 2016).

In the present study, the activity of human GST isoforms in the inactivation of AQ-QI and DEAQ-QI was studied using human liver microsomes (HLM) as bioactivation system. Next to the ten GSTs studied previously (Den Braver et al., 2016), also GSTK1-1 was included in the present study. GSTK1-1 is specifically localized in mitochondria which are considered critical targets organelles of many toxicants. GSTK1-1 shows high activity in the detoxification of byproducts of oxidative stress, such as peroxides and α,β-unsaturated aldehydes (Hayes et al., 2005). Whether GSTK1-1 can also catalyze GSH-conjugation of reactive drug metabolites has not been reported previously. The contribution of the most active GST isoform was further characterized at different GSH concentrations. Moreover, hepatic contents of GSTs of 22 individuals from literature were employed to simulate the inter-individual variability in GST-catalyzed inactivation of AQ-QI and DEAQ-QI in human liver cytosol. Additionally, the relative contributions of GSTs and NQO1 in inactivating AQ-QI and DEAQ-QI was investigated. Our results suggest that both GSTs and NQO1 may play important roles in the detoxification of the reactive QIs from AQ and DEAQ, via conjugation and reduction pathways, respectively. Inter-individual variability of expression levels of GSTs and NQO1 are suggested to have a significant influence on inactivation of AQ and DEAQ reactive metabolites.

2. Materials and methods

2.1. Materials

Amodiaquine dihydrochloride was obtained from INC Biomedicals (Aurora, OH, United States), N-Desethylamodiaquine was obtained from BD Biosciences (Franklin Lakes, NJ, United States). AQ-SG and DEAQ-SG references were synthesized and quantified as described previously (Zhang et al., 2017). Human liver microsomes (Lot No. 1210347), pooled from 200 donors, were purchased from Xenotech (Lenexa, United States). The expression plasmids for human GSTA1-1, GSTM1-1, and GSTP1-1 were kindly supplied by Prof. Mannervik (Department of Neurochemistry, Stockholm University, Sweden). The expression plasmid for GSTT1-1 was kindly provided by Prof. Hayes (Biomedical Research Centre, University of Dundee, Scotland, United Kingdom). Expression plasmids for GSTA2-2, GSTA3-3, GSTA4-4, GSTM2-2, GSTM3-3, GSTM4-4, GSTP1-1 allele B, C, and D, GSTT2-2, and NQO1 were constructed as described previously elsewhere (Venkataraman et al., 2014; Den Braver et al., 2016; Dekker et al., 2016; Vredenburg et al., 2014).

All other chemicals and reagents were of analytical grade and obtained from standard suppliers.

2.2. Construction of plasmids expressing human glutathione S-transferase K1-1 gene

The pCMV6-Entry plasmid carrying the human GSTK1-1 gene was purchased from Origene (Rockville, United States). The following primers containing restriction sites of NdeI and EcoRI (in bold) were used to amplify the coding region of the gene.

GSTK1-1 Forward: 5′-AAAA**CATATG**GGGCCCCTGCCG-3′

GSTK1-1 Reverse: 5′-AAAA**GAATTC**GCAAGTCTGGCATTCACGGC-3′

The amplified gene was subcloned into the pET-20b(+) vector obtained from Novagen (Madison, WI, United States) and a histidine-tag encoding gene was incorporated in the ORF to facilitate subsequent purification via nickel-agarose affinity chromatography. The final construct was transformed into competent *E. coli* BL21 cells. The correct sequences were verified by DNA sequencing (Macrogen, Amsterdam, the Netherlands).

2.3. Expression and purification of recombinant human GSTs and NQO1

Expression and purification of human GSTs were performed as described previously (Dragovic et al., 2010). As summary, *E. coli* BL21 cells transformed with the expression plasmids encoding GSTA1-1, GSTA2-2, GSTA3-3, GSTA4-4, GSTM1-1, GSTM2-2, GSTM3-3, GSTM4-4, GSTP1 wild type (*A) and alleles *B, *C, *D. Cells were cultured in 2YT-medium at 37 °C and the expression of protein was induced with 0.2 mM isopropyl β-D-1-thiogalactopyranoside (IPTG) after OD_{600} of the cultures reached 0.3. The purification of these recombinant human GSTs was performed with GSH-sepharose 4 B (GE Healthcare Life Sciences, Eindhoven, the Netherlands) affinity chromatography. The bacterial cells transformed with GSTT1-1, GSTT2-2, and GSTK1-1 genes were cultured in LB-medium at 37 °C and the expression was induced with 0.5 mM IPTG until OD_{600} reached 0.7. The purification of these

his-tagged recombinant human GSTs was performed with Ni-NTA (Sigma-Aldrich, Zwijndrecht, the Netherlands) affinity chromatography. Expression and purification of NQO1 was performed as described by Vredenburg et al. (2014).

The purity of the GST proteins was assessed by SDS-PAGE on a 15% gel following a Bio-Safe™ Coomassie staining (Bio-Rad, Hercules, CA, United States). Protein concentrations were determined using the method from Bradford (1976) with Quick Start™ Bradford 1X Dye Reagent (Bio-Rad, Hercules, CA, United States). Specific activities of the expressed recombinant human GSTs were determined spectrophotometrically using an Ultrospec 2000 UV/visible spectrophotometer (Pharmacia Biotech, Cambridge, England) and by using 1-chloro-2,4-dinitrobenzene (CDNB), 1,2-epoxy-3-(*p*-nitrophenoxy)propane (EPNP) and cumene hydroperoxide as substrates. Specific activities of all recombinant human GSTs, except GSTT1-1 and GSTT2-2, were measured using 400 μM of CDNB and 1 mM GSH as substrates, according to the method of Habig et al. (1974). For human GSTT1-1, 500 μM EPNP and 5 mM GSH were used as the substrates as previously described (Jemth and Mannervik, 1997). For GSTT2-2, GSH peroxidase activity using cumene hydroperoxide as substrate was determined with minor modification from the method of Prohaska et al. (1977). This assay contains 100 mM potassium phosphate buffer, pH 7.4, 5 mM EDTA, 4 μg/mL GSH reductase, 0.3 mM NADPH, and 1 mM GSH. The reaction was initiated with the addition of cumene hydroperoxide at a concentration of 1 mM. The linear decrease of absorbance at 340 nm, representing the oxidation of NADPH, was measured. The difference in the molar extinction coefficient (Δe) was 6.22 mM^{-1} cm^{-1} as reported previously (Habig et al., 1976). Specific activity of NQO1 was determined using 2,6-dichlorophenolindophenol (DCPIP) as substrate as described previously (Siegel et al., 2004).

2.4. Activity of recombinant human GSTs in the inactivation of reactive QIs of AQ and DEAQ using pooled HLM as bioactivation system

AQ (100 μM) and DEAQ (50 μM) were incubated with HLM at a final microsomal protein concentration of 1 mg/mL in 100 mM potassium phosphate buffer (KPi buffer, pH 7.4, containing 2 mM EDTA and 5 mM $MgCl_2$). These concentrations of AQ and DEAQ were chosen based on the enzyme kinetical study of AQ-SG and DEAQ-SG formation in HLM, showing near maximal bioactivation of AQ and DEAQ (Zhang et al., 2017). To investigate the role of human GSTs under low and high GSH concentrations, 0.5 mM and 5 mM GSH were used in the absence and presence of 8 μM recombinant human GST isoforms. These two GSH concentration conditions were selected to represent normal physiological situation and oxidative stress situation. A concentration of 8 μM of recombinant GST was used to be within the range of reported concentrations of hepatic GSTs (Van Ommen et al., 1990) and which was found to show significant increases in GSH-conjugation of AQ-QI and DEAQ-QI. Reactions were initiated by adding NADPH regenerating system (NRS; containing final concentrations of 0.1 mM NADPH, 10 mM glucose-6-phosphate, and 0.5 Units/mL glucose-6-phosphate dehydrogenases). All incubations were conducted at a final volume of 100 μL and at 37 °C in duplicate. Incubations were proceeded for 10 and 45 mins for AQ and DEAQ respectively, considering the linearity of product formation over these time periods (Zhang et al., 2017). Reactions were terminated by adding ice-cold perchloric acid at a final concentration of 1% (*v/v*). Incubation samples were cooled on ice for 10 mins and centrifuged for 15 mins at 14000 rpm. The supernatants were analyzed by HPLC-UV, as described in Section 2.7.

To investigate the possible contribution of microsomal GST to GSH-conjugation of reactive quinoneimines of AQ and DEAQ, incubations were also performed in presence of 500 μM S-hexyl glutathione, a potent inhibitor of both cytosolic and microsomal GSTs, as described by Mosialou and Morgenstern (1990).

For the most active recombinant human GST isoform, GSTP1-1*A, the enzymatic GSH-conjugation of reactive QIs derived from AQ and DEAQ were investigated further in the presence of seven different GSH concentrations (0.05, 0.1, 0.2, 0.5, 1, 2 and 5 mM). Non-enzymatic conjugation at these GSH concentrations was studied in the absence of GSTs. All the other incubation conditions were identical as described above.

2.5. Simulation of inter-individual differences of GSH-dependent inactivation of AQ-QI and DEAQ-QI by human liver cytosol

To investigate the inter-individual variations of GST-catalyzed inactivation of reactive QIs from AQ and DEAQ in human liver cytosol, the same methodology as described by Den Braver et al. (2016) was employed in the current study. The hepatic GST contents from 22 donors were taken as published by Van Ommen et al. (1990) and Rowe et al. (1997). Although relatively high GSTP1-1 concentrations were reported in these two studies, these were not included in the present simulation, since immunohistochemical studies have shown that expression of GSTP1-1 appears mainly restricted to the bile duct whereas only traces were found in hepatocytes (Lakehal et al., 1999; Desmots et al., 2001). Total individual GST activities in catalyzing the inactivation of AQ-QI and DEAQI-QI were calculated by multiplying hepatic contents of GSTs and specific activities of corresponding recombinant GSTs at 0.5 and 5 mM GSH. Each hepatic GST activity was normalized to the non-enzymatic contribution measured under their corresponding GSH concentration.

2.6. Competition between NQO1 reduction of AQ-QI and DEAQ-QI and GSTP1-1 catalyzed GSH conjugation

To investigate whether NQO1 was able to catalyze the reduction of AQ-QI and DEAQ-QI and to compare the relative catalytic activity of NQO1 with GST, incubations of AQ and DEAQ with 1 μM GSTP1-1*A and 1 μM NQO1 were performed as described in Section 2.4. Incubations containing 50 μM of dicoumarol were performed to investigate whether reduced GSH-conjugate formation in presence of NQO1 was due to inhibition of catalytic activity of NQO1 or due to covalent binding of the QIs to the NQO1 protein. Control incubations in the absence of GSTP1-1*A or NQO1 were performed. The GSH concentration was set at 100 μM to minimize the contribution of non-enzymatic GSH-conjugation.

2.7. Analytical methods

The separation and quantification of AQ and DEAQ and their metabolites were performed as described previously (Zhang et al., 2017). In brief, samples were analyzed by a reverse-phase liquid chromatography. The Shimadzu HPLC-system consisted of a SIL-20AC auto-sampler cooled at 4 °C, two LC20AD binary pumps and a SPD-20A UV/VIS detector (Shimadzu, Kyoto, Japan). Chromatographic separation was performed by using a Luna 5 μm C18 column (4.6 mm × 150 mm) as stationary phase, protected by a 4.0 mm × 3.0 mm i.d. security guard (5 μm) C18 guard column, both from Phenomenex (Torrance, CA). An isocratic method (9% acetonitrile in 5 mM of ammonium acetate buffer, pH 2.4, adjusted by formic acid) was used for separation of AQ and DEAQ and their metabolites. The flow rate used was 0.75 mL/min and the detector was set at 342 nm. Standard curves were constructed for AQ-SG (ranging from 0.05 to 5 μM) and DEAQ-SG (ranging from 0.01 to 5 μM). Data analysis was performed using the Shimadzu LC solution software, Version 1.24 SP1.

Table 1
Specific activities of recombinant human GSTs and human NQO1.

Enzymes	Substrates	Specific activity[a] (μmol/min/mg protein)
GSTA1-1	CDNB (0,4 mM); GSH (1 mM)	13.7
GSTA2-2	CDNB (0,4 mM); GSH (1 mM)	2.07
GSTA3-3	CDNB (0,4 mM); GSH (1 mM)	3.90
GSTA4-4	CDNB (0,4 mM); GSH (1 mM)	9.49
GSTM1-1	CDNB (0,4 mM); GSH (1 mM)	30.6
GSTM2-2	CDNB (0,4 mM); GSH (1 mM)	30.5
GSTM3-3	CDNB (0,4 mM); GSH (1 mM)	1.29
GSTM4-4	CDNB (0,4 mM); GSH (1 mM)	3.69
GSTP1-1*A	CDNB (0,4 mM); GSH (1 mM)	23.3
GSTP1-1*B	CDNB (0,4 mM); GSH (1 mM)	22.3
GSTP1-1*C	CDNB (0,4 mM); GSH (1 mM)	21.3
GSTP1-1*D	CDNB (0,4 mM); GSH (1 mM)	44.3
GSTK1-1	CDNB (0,4 mM); GSH (1 mM)	1.25
GSTT1-1	EPNP (0,5 mM); GSH (5 mM)	1.35
GSTT2-2	Cumene hydroperoxide (1 mM); GSH (1 mM)	1.25
NQO1	DCPIP (40 μM)	280

[a] Activity was average values measured from two independent incubations. Variation was less than 5%.

3. Results

3.1. Activity of recombinant human GSTs in the inactivation of reactive QIs of AQ and DEAQ using HLM as bioactivation system

3.1.1. Expression and purification of recombinant human GST isoforms and NQO1

The purity of the isolated recombinant human GST isoforms and human NQO1 was all above 90% as assessed by SDS-PAGE. The specific activities of the purified enzymes are tabulated in Table 1 and are consistent with published data (Siegel et al., 2004; Dekker et al., 2017; Siegel et al., 2004).

3.1.2. Metabolic profiles of AQ and DEAQ in HLM

After incubating AQ and DEAQ respectively with HLM in the presence of GSH under the current incubation conditions, DEAQ and AQ-SG appeared to the two major metabolites formed in AQ incubations while DEAQ-SG and N-bisdesethylamodiaquine (bis-DEAQ) were the two major metabolites formed in DEAQ incubations (Supplemental Fig. S1). Because of the short incubation time, no secondary metabolites of DEAQ, such as bis-DEAQ and DEAQ-SG, were observed in AQ incubations. The metabolic profiles of AQ and DEAQ are consistent with described by Zhang et al. (2017). Addition of GSTs did only show increase of the amount of previously identified AQ-SG and DEAQ-SG, indicating that no change in regioselectivity of GSH-conjugation is applicable, as was observed previously for the reactive QIs of diclofenac (Den Braver et al., 2016).

3.1.3. Activity of recombinant human GSTs in the inactivation of AQ-QI and DEAQ-QI

To investigate the activity of individual human GSTs in the inactivation of reactive QIs of AQ and DEAQ, 100 μM AQ and 50 μM DEAQ were incubated with 8 μM recombinant human GSTs in the presence of 0.5 and 5 mM GSH. These concentrations of AQ and DEAQ did not inhibit GST activity when tested with standard substrates (Supplemental Fig. S2). Addition of 500 μM S-hexyl-glutathione to the microsomal incubations of AQ and DEAQ with 0.5 or 5 mM GSH did not show any effect on the amount of formation of AQ-SG and DEAQ-SG, Supplemental Fig. S5. This concentration of S-hexyl glutathione inhibited GSH-conjugation of CDNB by the pooled HLM by 90%, Supplemental Fig. S4. Therefore, microsomal GST or residual cytosolic GSTs in HLM do not seem to be involved in the formation of AQ-SG and DEAQ-SG in absence of recombinant human GSTs.

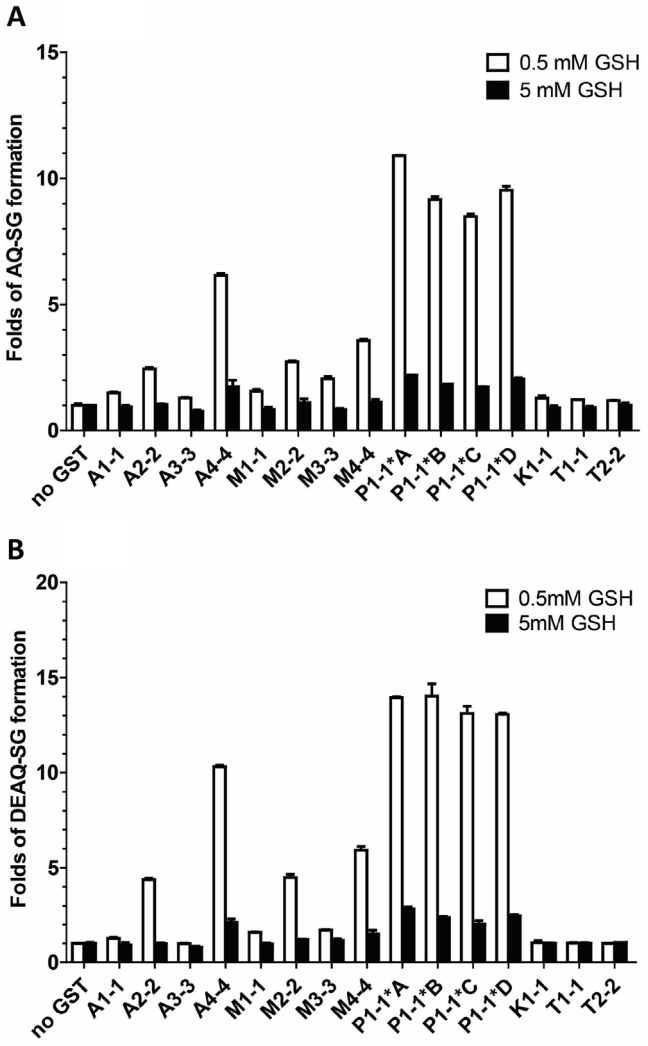

Fig. 1. Effects of recombinant human glutathione S-transferases (GSTs, 8 μM) on the inactivation of Amodiaquine quinoneimine (A) and N-desethylamodiaquine quinoneimine (B). Data are expressed as folds difference relative to non-enzymatic conjugations. Incubations were performed in the presence of 1 mg/mL HLM and an NADPH regenerating system as described in detail in Materials and Methods section. Each bar represents average ± range from two separate incubations.

As shown in Fig. 1A, at 0.5 mM GSH, recombinant human GST P1-1 and its alleles were the most active isoforms for the inactivation of AQ-QI, by forming 8.5- to 10.9-fold higher levels of AQ-SG compared to the non-enzymatic conjugation. GST isoforms A4-4, M4-4, M2-2, and A2-2 also showed significant increase of AQ-SG formation by forming 6.1-, 3.6-, 2.7-, and 2.4-fold higher concentrations, respectively, compared with non-enzymatic conjugation. Incubation with the other GST isoforms showed levels of GSH-conjugates less than 2-fold of that in absence of GSTs. At 5 mM GSH the relative increase by the human GST isoforms was reduced due to the stronger non-enzymatic contribution. At this high GSH concentration, GSTP1-1 and its variants, GSTA4-4, GSTM4-4, GSTM2-2, GSTA2-2 showed 1.7- to 2.2-, 1.8-, 1.1-, 1.1-, 1.2-fold of GSH-conjugates formed compared to non-enzymatic conjugation, respectively.

In case of DEAQ, comparable activities in the inactivation of DEAQ-QI by GSTP1-1 and its alleles were observed at 0.5 mM GSH compared to the same condition of AQ (Fig. 1B), with exhibiting 13.0- to 13.9-fold higher levels of DEAQ-SG compared to non-enzymatic conjugation, respectively. GSTA4-4, GSTM4-4, GSTM2-2, and GSTA2-2 showed significant increases of 10.3-, 5.9-, 4.5-, and 4.4-fold and the rest GST

Table 2
Concentrations of GSH-conjugates formed in incubations of AQ and DEAQ with human liver microsomes and GSH, in absence and presence of recombinant human glutathione S-transferase (GST).

	AQ-SG (μM)[a,c]		DEAQ-SG (μM)[b,c]	
	0.5 mM GSH	5 mM GSH	0.5 mM GSH	5 mM GSH
No GST	0.17 ± 0.02	0.84 ± 0.01	0.09 ± 0.01	0.52 ± 0.03
GSTA1-1	0.25 ± 0.01	0.88 ± 0.02	0.11 ± 0.01	0.50 ± 0.10
GSTA2-2	0.40 ± 0.02	0.98 ± 0.03	0.56 ± 0.03	0.68 ± 0.04
GSTA3-3	0.22 ± 0.01	0.60 ± 0.07	0.09 ± 0.01	0.44 ± 0.04
GSTA4-4	1.02 ± 0.02	1.56 ± 0.37	0.88 ± 0.01	1.10 ± 0.13
GSTM1-1	0.26 ± 0.02	0.68 ± 0.11	0.14 ± 0.01	0.53 ± 0.04
GSTM2-2	0.45 ± 0.02	0.95 ± 0.21	0.39 ± 0.02	0.65 ± 0.02
GSTM3-3	0.34 ± 0.02	0.67 ± 0.06	0.15 ± 0.01	0.63 ± 0.06
GSTM4-4	0.59 ± 0.01	0.98 ± 0.15	0.51 ± 0.02	0.79 ± 0.13
GSTP1-1*A	1.80 ± 0.01	2.00 ± 0.02	1.19 ± 0.01	1.46 ± 0.07
GSTP1-1*B	1.51 ± 0.03	1.67 ± 0.01	1.20 ± 0.08	1.23 ± 0.04
GSTP1-1*C	1.40 ± 0.02	1.56 ± 0.01	1.12 ± 0.05	1.05 ± 0.13
GSTP1-1*D	1.58 ± 0.04	1.85 ± 0.07	1.11 ± 0.01	1.28 ± 0.05
GSTK1-1	0.21 ± 0.01	0.74 ± 0.11	0.09 ± 0.02	0.54 ± 0.02
GSTT1-1	0.20 ± 0.01	0.76 ± 0.05	0.09 ± 0.01	0.54 ± 0.04
GSTT2-2	0.20 ± 0.01	0.84 ± 0.14	0.09 ± 0.01	0.58 ± 0.01

[a] AQ (100 μM) was incubated for 10 min in the presence of 1 mg/mL HLM, an NADPH regenerating system, 0.5 mM or 5 mM GSH and 8 μM recombinant GST.
[b] DEAQ (50 μM) was incubated for 45 min in the presence of 1 mg/mL HLM, an NADPH regenerating system, 0.5 mM or 5 mM GSH and 8 μM recombinant GST.
[c] Data are expressed as average ± range from two separate experiments.

isoforms showed less than 2-fold increase to non-enzymatic conjugation. At 5 mM GSH concentration, only GST P1-1 and its alleles and A4-4 showed over 2-fold increase while the other GST isoforms showed less than 1.5-fold increase in DEAQ-SG formation, since the non-enzymatic conjugation was stronger under this condition. The absolute concentrations of AQ-SG and DEAQ-SG formed in this experiment were shown in Table 2.

3.2. GSH-dependent inactivation of non-enzymatic and GST-catalyzed conjugation of reactive QIs of AQ and DEAQ

To further characterize the GST-catalyzed inactivation of reactive QIs of AQ and DEAQ, the most active isoform GSTP1-1 (8 μM) was incubated with AQ and DEAQ with seven GSH concentrations varying from 0.05 to 5 mM. As shown in Fig. 2A, in absence of cytosolic GSTs, the conjugation of AQ-QI was linear up to GSH concentration of 1 mM, while showing a saturation tendency when GSH concentration was higher than 1 mM. The maximal formation of AQ-SG under this condition was 0.84 μM. Addition of 500 μM S-hexyl glutathione, which completely abolished microsomal GSH-conjugation of CDNB, did not affect the formation of AQ-SG (Figs. S4 and S5). Therefore, the apparent saturation does not result from saturation of microsomal GST but may result from near-maximal inactivation of reactive QIs by GSH-conjugation or GSH-oxidation. In the presence of GSTP1-1, AQ-SG formation at each GSH concentration was significantly increased compared to the corresponding non-enzymatic counterpart. The maximal formation of AQ-SG was reached at 0.5 mM GSH and higher GSH concentrations, with generating around 2 μM AQ-SG.

For the GSH-dependent formation of DEAQ-SG, as shown in Fig. 2B, comparable results were obtained. In the presence of GSTP1-1, the total DEAQ-SG formation was significantly increased when compared to incubations without GSTP1-1. Maximal DEAQ-SG formation observed in GSTP1-1 presenting situation was 1.65 μM (at 0.5 mM GSH) while in GSTP1-1 absent situation was 0.5 μM (at 5 mM GSH).

3.3. Simulation of inter-individual variation of GST-catalyzed inactivation of AQ-QI and DEAQ-QI by human liver cytosol

To simulate the inter-individual variability of GST-catalyzed conjugation of reactive QIs from AQ and DEAQ in human liver cytosol,

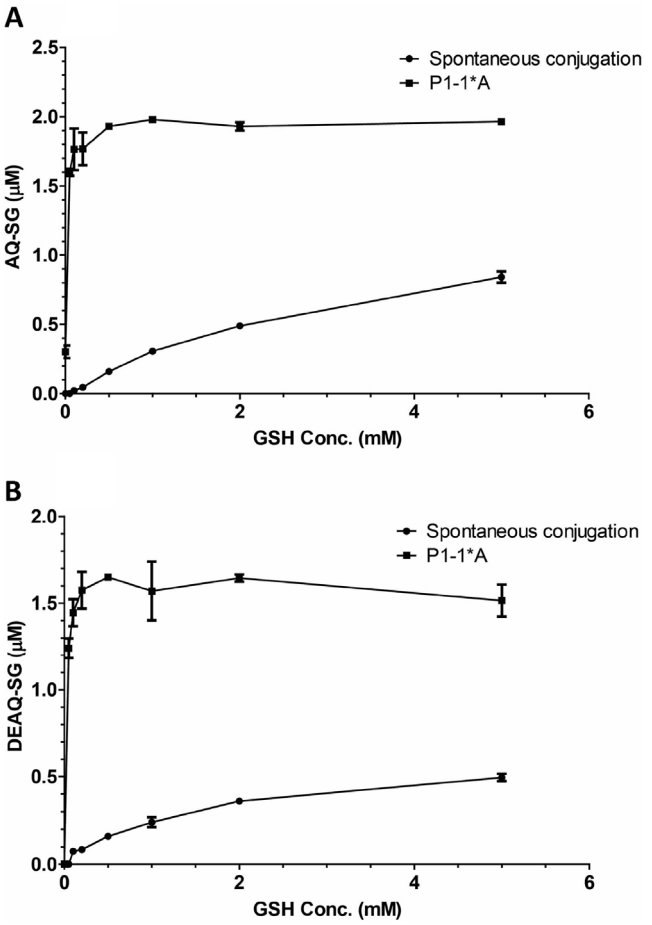

Fig. 2. GSH dependency curves of AQ-SG (A) and DEAQ-SG (B) formation in the absence and presence of GSTP1-1*A (8 μM) in incubations with 100 μM AQ and 50 μM DEAQ, respectively. Incubations were performed in the presence of 1 mg/mL HLM and an NADPH regenerating system as described in detail in Materials and Methods section. Data are presented as average ± range of two separate incubations. Lines do not represent fits to data and are only intended to guide the eyes.

enzymatic activities of recombinant GSTs in the formation of AQ-SG and DEAQ-SG under 0.5 mM and 5 mM GSH were calculated, as shown in Table 2. The activity of hepatic GST used in the simulation was linear with enzyme concentration in the range of concentrations among 22 donors, see Supplemental Fig. S3. With the normalization of non-enzymatic GSH-conjugation to one-fold for all situations, over-all conjugation and the contribution from each hepatic GST isoform of the formation of AQ-SG and DEAQ-SG were shown in Fig. 3. Large inter-individual variations of GST-catalyzed inactivation of AQ-QI (Fig. 3A) and DEAQ-QI (Fig. 3B) were observed under 0.5 mM GSH. For the inactivation of AQ-QI, a variation from 5.4-fold (Donor 11) to 31.8-fold (Donor 10) was observed while for the inactivation of DEAQ-QI, a variation from 6.8-fold (Donor 14) to 59.1-fold (Donor 10) was observed. These variations were mainly due to low chemical contribution and high GSTA2-2 activity under this GSH concentration. Although GSTA1-1 is the most abundant GST isoforms in human liver, because of its relatively low activity in catalyzing the formation of AQ-SG and DEAQ-SG, the contribution to this GST isoform under 0.5 mM GSH is still minor. In addition, GSTM1-1, the isoform which did not express in half of donors in this data set, barely contributed to the variation because of its low catalytic activity.

At 5 mM GSH, the inter-individual variability of inactivation of AQ-QI and DEAQ-QI by GSH-conjugation was much smaller compared to that of 0.5 mM GSH situation, due to the increased contribution of non-enzymatic conjugation, as shown in Fig. 3C and D. Under this condition, 1.5-fold (Donor 11 and Donor 12) to 4.7-fold (Donor 10)

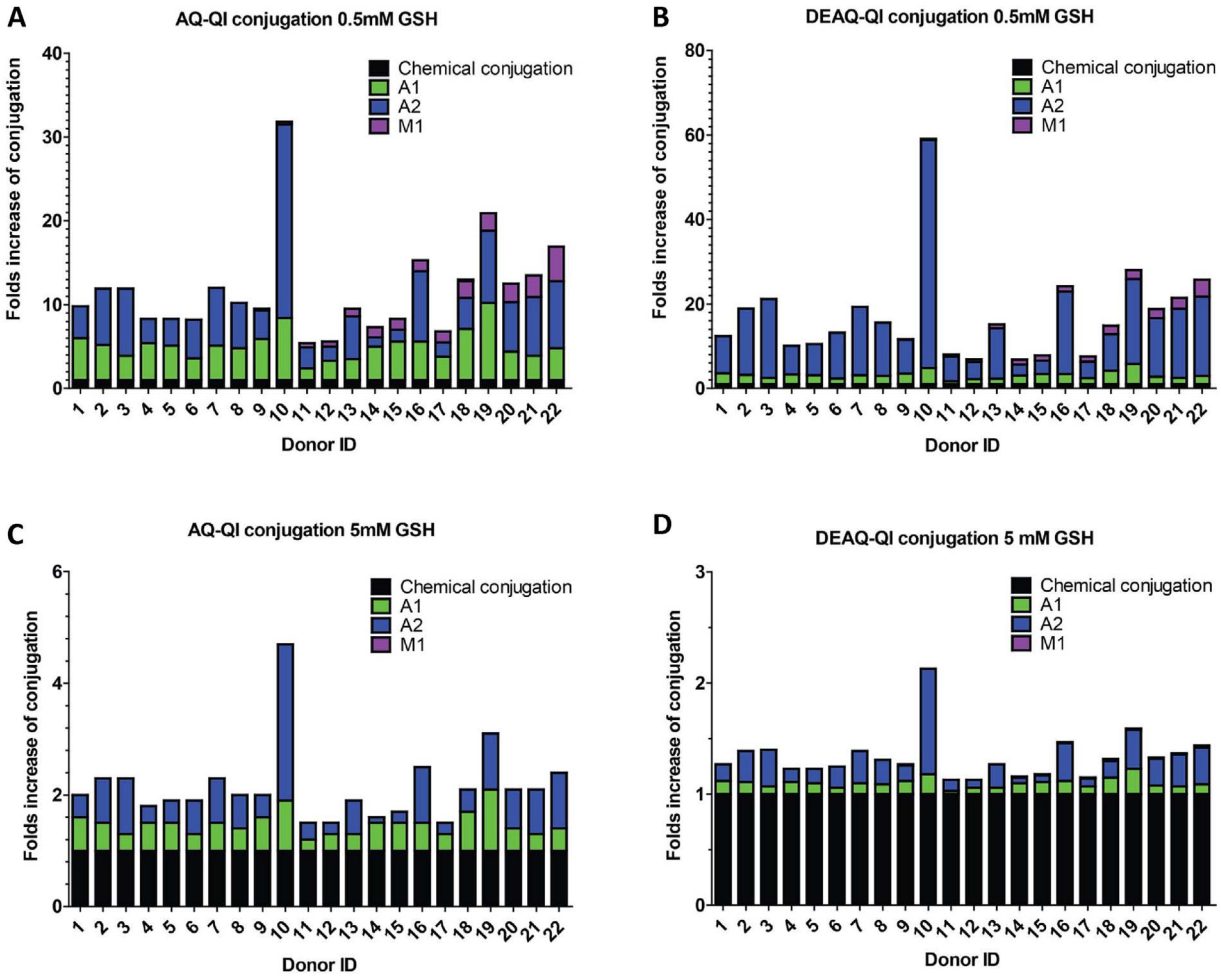

Fig. 3. Simulation of inter-individual variability of GSH-conjugation of reactive Quinoneimines from Amodiaquine and N-Desethylamodiaquine in human liver cytosol. Data are expressed as folds increase of GSH conjugation of each donor relative to non-enzymatic contribution (set as 1 fold). Hepatic GST contents and sorting of donors are adapted from Den Braver et al. (2016) and the activity of each GST isoform is measured in the present study. (A) Variation of GSH conjugation of AQ-QI at 0.5 mM GSH; (B) Variation of GSH conjugation of DEAQ-QI at 0.5 mM GSH; (C) Variation of GSH conjugation of AQ-QI at 5 mM GSH; (D) Variation of GSH conjugation of DEAQ-QI at 5 mM GSH.

variation of AQ-QI inactivation and 1.1-fold (Donor 12) to 2.1-fold (Donor 10) variation of DEAQ-QI inactivation were observed in this small population. In addition to GSTA2-2, which contributed predominantly to the inter-individual variation of AQ-SG formation, the variation caused by GSTA1-1 also contributed significantly at 0.5 mM GSH (1.4-fold in Donor 11 to 9.2-fold in Donor 19).

3.4. Competition of human GSTs and NQO1 in the inactivation of reactive QIs of AQ and DEAQ

To investigate the activity of NQO1 in the reduction of reactive QIs of AQ and DEAQ, a competition experiment between NQO1-catalyzed reduction and GST-catalyzed conjugation was performed. 1 μM GSTP1-1*A was selected to compete with NQO1 in the inactivation of AQ-QI and DEAQ-QI since it is the GST isoform with the highest catalytic activity in the GSH-conjugation of AQ-QI and DEAQ-QI. As shown in Fig. 4, the presence of 1 μM NQO1 decreased the formation of AQ-SG and DEAQ-SG under both non-enzymatical GSH-conjugation and GSTP1-1*A catalyzed enzymatic conjugation situations. In the absence of GSTP1-1*A, the chemical GSH conjugation of AQ-QI and DEAQ-QI were decreased by 51% and 19%, respectively, by 1 μM NQO1. In the presence of 1 μM GSTP1-1*A, the enzymatic GSH-conjugation of AQ-QI and DEAQ-QI were decreased by 37% and 60% compared to incubations in absence of NQO1. The reduced formation of GSH-conjugates was demonstrated to be caused by the catalytic effect of NQO1, since the addition of dicoumarol reverted GSH-conjugate formation of AQ-SG

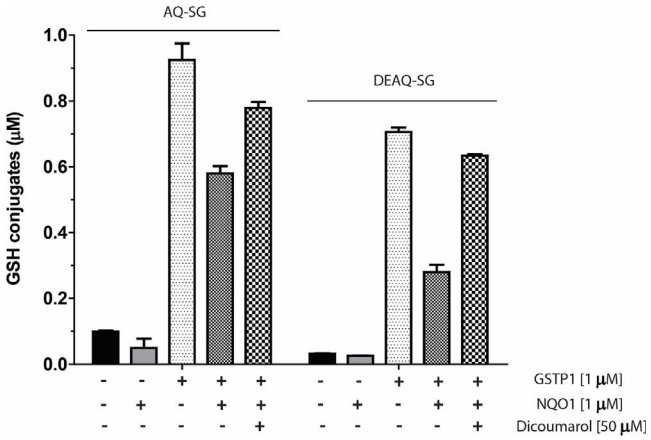

Fig. 4. Competition between GSH conjugation catalyzed by GSTP1-1*A and reduction catalyzed by NQO1 on the inactivation of AQ-QI and DEAQ-QI. Incubations were performed in the presence of 1 mg/mL HLM and an NADPH regenerating system as described in detail in Materials and Methods section. Y-axis shows the formation of GSH-conjugates and X-axis shows the experimental conditions. Bars represent average ± range from two separate incubations.

to 85% and DEAQ-SG to 90%.

4. Discussion

Oxidative metabolism leading to the formation of reactive metabolites has been suggested as an important mechanism underlying AQ-induced hepatotoxicity and agranulocytosis (Harrison et al., 1992; Naisbitt et al., 1998). Human cytochrome P450s, particularly CYP3A4, CYP2C8, CYP2D6, and CYP2C9 (Zhang et al., 2017), and myeloperoxidase (MPO) (Naisbitt et al., 1998; Lobach and Uetrecht, 2014) have been shown to play important roles in the bioactivation of AQ and DEAQ, a major oxidative metabolite of AQ. Together with oxidative bioactivation, which leads to the formation of two different reactive QIs, the rates of inactivation of the QIs catalyzed by human GSTs and NQO1 will also determine the internal exposure of an organ or tissue to the reactive QIs.

In the current study, the activities of 15 human GST isoforms in the GSH conjugation and the relative activity of NQO1 in the reduction of AQ-QI and DEAQ-QI were investigated. In addition, the potential inter-individual variability of GST-catalyzed detoxification profiles of AQ-QI and DEAQ-QI was studied, by applying previously published data set of major hepatic GST contents. Previous investigations showed that the cytotoxicity of AQ could be significantly reduced with a chemical antioxidant N-acetyl cysteine (Heidari et al., 2014), whereas GSH-depleted rat hepatocytes were more vulnerable upon AQ treatment (Tafazoli and O'Brien, 2009; Heidari et al., 2014). These studies mostly focused on the involvement of AQ-QI, in the context of AQ-induced cytotoxicity; however, the reactive metabolite of DEAQ has been overlooked. As the major circulating metabolite following AQ administration (Laurent et al., 1993; Lai et al., 2009; Scarsi et al., 2014), DEAQ and its reactive metabolite, DEAQ-QI, might be more important determining the internal exposure of individuals to reactive toxic metabolites (Zhang et al., 2017).

As shown in Fig. 1 and Table 2, several GSTs appeared to be active in catalyzing the GSH-conjugation of AQ-QI and DEAQ-QI. GSTP1-1 and GSTA4-4 showed the highest activities in the formation of AQ-SG and DEAQ-SG, especially under GSH-depleted conditions, whereas GSTA1-1 and GSTM1-1, constituting the major hepatic GST isoforms showed very low activity in this process. Additionally, hepatic GSTA2-2 and extra-hepatic GSTM4-4 and GSTM2-2 showed intermediate GSH-conjugation activities leading to the inactivation of both QIs. In absence of cytosolic GSTs, the formation of AQ-SG and DEAQ-SG in HLM appeared to be formed non-enzymatically since addition of S-hexylglutathione, which can inhibit both cytosolic and microsomal GST (Mosialou and Morgenstern, 1990), did not reduce formation of GSH-conjugates (Fig. S4). Considering the fact that by using HLM as bioactivating system the steady-state concentration of the QIs during the incubation are most likely far below the corresponding K_m-values, the different yields of GSH-conjugates will be a reflection of the different intrinsic clearances (k_{cat}/K_m) of the enzymes. Because for the more active GSTs, the steady-state concentration of the QIs will also be lower, their k_{cat}/K_m-values would even be underestimated when only considering the concentration of GSH-conjugates.

The most active GSTs, GSTP1-1 and GSTA4-4, are known to be highly expressed in bile ducts in the liver, and in skin and heart tissues (Lakehal et al., 1999; Desmots et al., 2001), respectively. Nevertheless, in isolated human hepatocytes both GSTP1-1 and GSTA4-4 could be detected at low levels when compared to the most abundant GSTA1-1 (Sison-Young et al., 2015; Desmots et al., 2001). Gallagher et al. (2006) reported the presence of GSTP1-1, GSTA1-1, GSTA2-2 and GSTA4-4 in mitochondria of human hepatocytes. In addition, GSTK1-1 has been demonstrated to be specifically localized in mitochondria (Jowsey et al., 2003). Collectively, these studies suggest that various GSTs may play an important role in protecting hepatic tissues from the damage by reactive metabolites and in maintaining a favorable mitochondrial redox state. However, since the GST isoforms in human blood cells and bone marrow are not fully characterized yet, the correlation of blood GSTs with AQ-induced agranulocytosis warrants further research.

As shown in Fig. 2, the total amount of GSH-conjugates of AQ-QI and DEAQ-QI in the presence of GSTP1-1 strongly increased even at the highest GSH concentration. This observation indicated that a significant amount of the QIs formed at low GSH-concentration, and in absence of additional GSTs undergo different reactions, since no QIs were detected directly by HPLC. Next to GSH-conjugation, alternative reactions of the QIs include GSH-oxidation (regenerating drug and producing GSSG), oxidation of NADPH, and reaction with microsomal proteins by covalent binding or protein oxidation. Previous study reported that the ratio of reduction versus GSH-conjugation of reactive metabolite of acetaminophen, NAPQI, significantly shifted to GSH-conjugation side when adding 0.1 mg/mL GSTP1-1 into the incubations (Coles et al., 1988). Therefore the reactions of AQ-QI and DEAQ-QI with GSH might also shift from GSH-oxidation to GSH-conjugation in the presence of GSTP1-1, which results the overall higher formation of GSH-conjugates. To what extent the addition of GSTs influence the degree of protein modification, however, remains to be established.

Recently, Den Braver et al. (2016) simulated the inter-individual variability of hepatic cytosolic GST-catalyzed inactivation of reactive QIs derived from diclofenac by multiplying the activities of major hepatic GST with their corresponding GST contents in 20 liver donors, assuming that rates of inactivation is proportional to GST concentrations. In the present study, a similar methodology was applied to study inter-individual variability of GST-catalyzed inactivation of AQ-QI and DEAQ-QI using the same published profiles of major human hepatic GSTs. Similar to the diclofenac study, large variations of GSH conjugation profiles of AQ-QI and DEAQ-QI were found among the 22 liver donors, as presented in Fig. 3. At 5 mM GSH concentrations, an up to 5-fold difference was observed for GSH conjugation of AQ-QI and DEAQ-QI and this difference is up to 59-fold at reduced GSH concentrations (500 μM) e.g. reflecting oxidative stress conditions. The large variation was predominantly caused by the varying expression levels of GSTA2-2 in this small population, since GSTA2-2 exhibited a moderate activity in the inactivation of AQ-QI and DEAQ-QI under both high and low GSH concentrations. Although this study showed that GSTP1-1 and GSTA4-4 were the most active enzymes, they were not included in the simulation study since no quantitative information is available about their concentrations in human hepatocytes. The relatively high levels of GSTP1-1 in the liver homogenates, as reported by Van Ommen et al. (1990) may be mainly originate from bile duct cells. In the HPLC-chromatograms of the GSTs obtained after affinity chromatography no peak was assigned as GSTA4-4, so their levels and inter-individual variability remains to be quantified.

Although previous association studies suggested increased risks of NSAID-induced liver injury (Lucena et al., 2008) and nevirapine-induced hepatotoxicity (Singh et al., 2015) for individuals carrying GSTM1 and GSTT1 double-null genotypes, in the present study, these two GST isoforms did not show significant catalytic activity regarding the GSH-conjugation of AQ-QI and DEAQ-QI. Thus the overall GSH-conjugation profiles were not different between GSTM1-negative and GSTM1-positive donors (Fig. 3). It is well known that inter-individual variation of clinical AQ pharmacokinetic parameters was large, for example, AUC values of AQ and DEAQ varied up to 4-fold in 42 patients during antimalarial therapy (Rijken et al., 2011). Thus, it can be reasonably assumed that individuals with high CYP-mediated bioactivation profiles combined with low inactivation profiles for AQ-QI and DEAQ-QI are potentially most susceptible to AQ-induced idiosyncratic hepatotoxicity.

In addition to human GSTs, in the present study NQO1 was showed to be able to catalyze the reductive inactivation of AQ-QI and DEAQ-QI, Fig. 4. The inhibition of NQO1-catalyzed reduction of AQ-QI and DEAQ-QI by dicoumarol confirmed the inactivation effects of NQO1. However, the activity of NQO1 appeared to be respectively 37% and

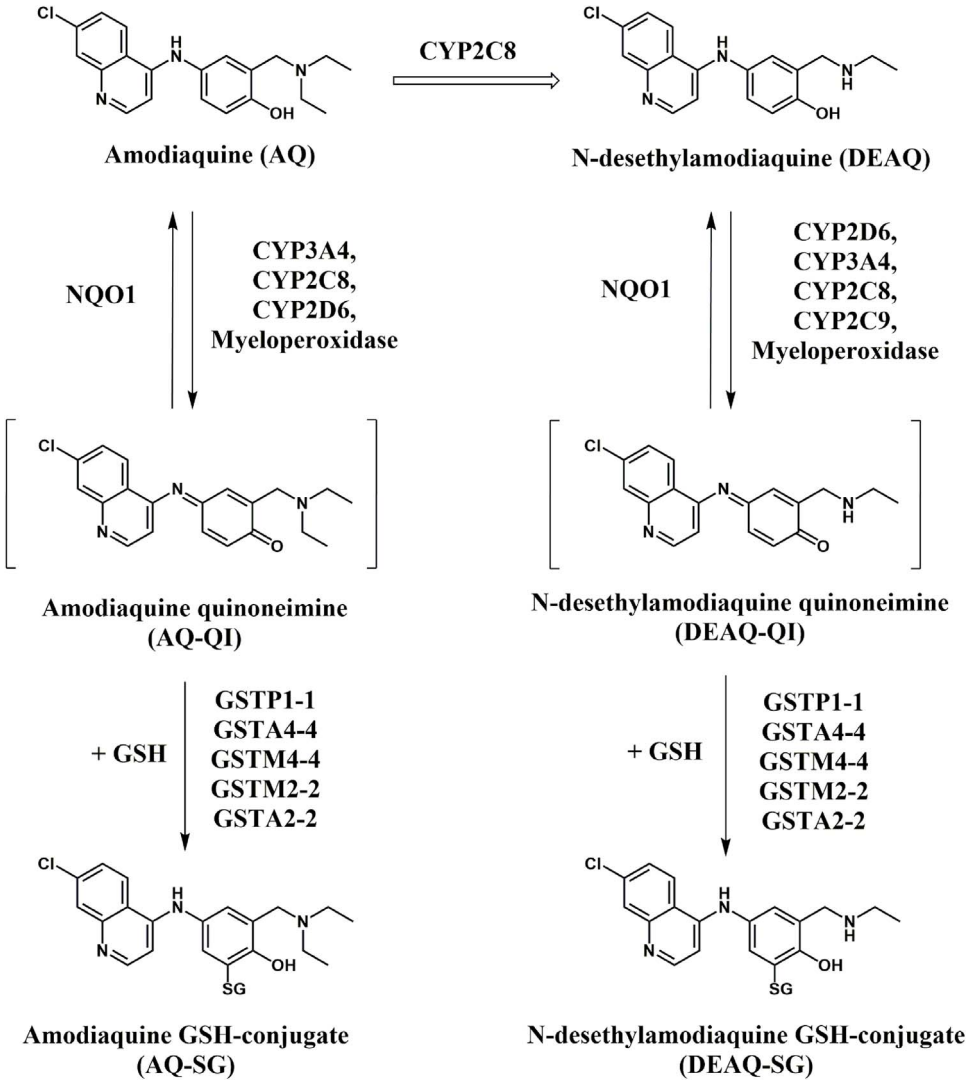

Fig. 5. Scheme of bioactivation by CYPs and MPO and inactivation by GSTs and NQO1scheme of AQ and DEAQ. Isoforms of CYPs in bioactivation pathway and GSTs in inactivation pathway are sorted by corresponding activities in decreasing order.

60% of that of GSTP1-1 at the same concentrations. NQO1 expression is normally considered quite low in human liver (Siegel et al., 2000). Vredenburg et al. (2014) estimated the basal concentration of NQO1 in human liver and found it to be around 0.3 μM. However, this enzyme is highly induced under oxidative stress conditions, notably via the Keap1:Nrf2 signaling pathway (Walsh et al., 2014). Up until now, the absolute quantification of NQO1 has only been studied in lung and colon tissues (Tang et al., 2013) but not yet in liver. Although Rougée et al. (2016) reported a large variability in NQO1 expression levels in 117 human livers, the individual NQO1 levels of expression were not provided. Altogether, it is reasonable to speculate that NQO1 is an additional detoxification pathway for AQ-QI and DEAQ-QI next to GST-catalyzed GSH-conjugation, certainly under oxidative stress conditions.

In conclusion, the results of the present study, in which HLM were used to bioactivate AQ and DEAQ, clearly demonstrated the crucial roles that human GST isoforms (15 tested) and NQO1 (1 tested) are playing in the inactivation of the reactive QIs of AQ and DEAQ, via GSH conjugation and reduction, respectively (Fig. 5). Using the experimentally determined activities and levels of the various GST isoforms, a large inter-individual variability of GST-catalyzed conjugation of AQ-QI and DEAQ-QI was observed from a panel of 22 liver donors. NQO1 showed competing activity to GSTP1 in the inactivation of AQ-QI and DEAQ-QI. Especially at low GSH-concentrations GSH-conjugation will be strongly dependent on catalysis by GSTs, so the variability of GST-expression will have the highest consequences under conditions of reduced GSH-concentration, such as oxidative stress. Altogether, it can be assumed that individuals with low expression levels of GSTA2-2, GSTA1-1 and NQO1 in combination with high expression levels of bioactivating CYP or MPO enzymes, exposed to AQ and by inference to the major metabolite DEAQ, may be more susceptible to AQ idiosyncratic hepatotoxicity and agranulocytosis, because of higher levels of potentially antigenic protein-adducts.

Conflict of interest

None.

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Appendix A. Supplementary data

Supplementary data associated with this article can be found, in the online version, at http://dx.doi.org/10.1016/j.toxlet.2017.05.003.

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